BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTU Inter

INTERNATIONAL APPLICATION PUBLISHED

WO 9602650A2

(51) International Patent Classification 6 :
C12N 15/52, 15/82, A01H 5/00 A2

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/GB95/01603

(22) International Filing Date: 6 July 1995 (06.07.95)

(30) Priority Data:
9414505.9 18 July 1994 (18.07.94) GB
9419081.6 22 September 1994 (22.09.94) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KARVOUNI, Zoi [GR/GR]; Vioryl, 36 Viltaniotis Street, GR-145 64 Kifissia (GR). JOHN, Isaac [PK/GB]; University of Nottingham, Dept. of Physiology and Environmental Science, Sutton Bonington, Loughborough LE12 5RD (GB). TAYLOR, Jane [GB/GB]; Lancaster University, Institute of Environmental and Biological Sciences, Division of Biological Sciences, Lancaster LA1 4YQ (GB). WATSON, Colin [GB/US]; University of Arizona, Dept. of Plant Sciences, Tucson, AZ 85721 (US). TURNER, Andrew [GB/GB]; Scotgen Limited, 2 Lillydron Avenue, Aberdeen AB9 2TN (GB). GRIERSON, Donald [GB/GB]; University of Nottingham, School of Agriculture, Dept. of Plant Physiology, Sutton Bonington, Loughborough LE12 5RD (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG12 6YA (GB).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: DNA, CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

(57) Abstract

A DNA encoding melon phytoene synthase.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

DNA, CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

This application relates to novel DNA constructs, plant cells containing the constructs and plants derived therefrom. In particular it relates to the modification of plant gene expression causing modification of carotenoid metabolism in plants.

The modification of plant gene expression has been achieved by several methods. The molecular biologist can choose from a range of known methods to decrease or increase gene expression or to alter the spatial or temporal expression of a particular gene. For example, the expression of either specific antisense RNA or partial (truncated) sense RNA has been utilised to reduce the expression of various target genes in plants (as reviewed by Bird and Ray, 1991, Biotechnology and Genetic Engineering Reviews 9:207-227). These techniques involve the incorporation into the genome of the plant of a synthetic gene designed to express either antisense or sense RNA. They have been successfully used to down-regulate the expression of a range of individual genes involved in the development and ripening of tomato fruit (Gray et al, 1992, Plant Molecular Biology, 19:69-87). Methods to increase the expression of a target gene have also been developed. For example, additional genes designed to express RNA containing the complete coding region of the target gene may be incorporated into the genome of the plant to "over-express" the gene product. Various other methods to modify gene expression are known; for example, the use of alternative regulatory sequences.

Carotenoids include a range of biochemicals such as carotenes, lutein, xanthophylls, and pigments such as lycopene. The biosynthesis of carotenoids in higher plants and micro-organisms has been the subject of numerous investigations. The pathways of carotenoid biosynthesis across a range of organisms share many common features (Bramley and Mackenzie, 1988). The biochemical pathway leading to carotenoid biosynthesis is shown in Figure 1.

The divergence to the dedicated pathway for carotenoid synthesis is after the formation of geranylgeranyl diphosphate (GGDP - previously known as geranylgeranyl pyrophosphate, GGPP). The first unique step in the formation of carotenoids is the head-to-head condensation of 2 molecules of

GGDP to form the first C_{40} carotenoid, phytoene. The synthesis of phytoene from GGDP involves two reaction steps with prephytoene diphosphate (PPDP - previously known as prephytoene pyrophosphate, PPPP) as the intermediate, but both reactions are catalysed by a single enzyme in plants and micro-organisms. This enzyme, which catalyses the conversion of GGDP to phytoene, is known as phytoene synthase (Bramley et al, 1992, Plant Journal, 2:343-349).

Phytoene synthase activity has been detected in a wide range of plant species including tomato, pepper, maize, carrot, spinach and tobacco. There are many similarities between the enzymes from different species. The enzymes are located in the plastid stroma and synthesise the same phytoene sterio-isomer (15-cis phytoene). The enzymes all require Mn^{++} as a co-factor although the requirement for ATP is unclear.

DNA sequences encoding phytoene synthase enzymes are known. For example, the TOM5 gene encodes an enzyme involved in the ripening of tomatoes (Ray et al, 1987, Nucleic Acids Research, 15:10587) which has been identified as the enzyme phytoene synthase (international patent application published as WO91/09128; international patent application published as WO92/16635). Phytoene synthase enzymes show sequence similarity. The molecular weight of the purified pepper (Capsicum annuum) phytoene synthase (48kD) (described by Dogbo et al 1988) is very similar to the predicted molecular weight of the mature phytoene synthase protein encoded by TOM5 (47.6kD). Alignment of the predicted phytoene synthase protein sequences from Arabidopsis and pepper cDNA clones with the predicted protein sequence of the tomato phytoene synthase encoded by TOM5 shows 75% and 90.6% identity respectively. The protein encoded by the tomato TOM5 cDNA also shows significant homology (32% identity; 56.5% similarity) to the CrtB protein from Rhodobacter capsulatus, a gram-negative purple bacterium (Armstrong et al, 1990, JBC, 265:8329-8338). The CrtB protein catalyses the conversion of GGDP to phytoene (Bartley GE et al, 1992, J Biological Chem, 267:5036-5039).

There is homology between plant phytoene synthase DNA sequences. Tomato contains at least one other locus with homology to TOM5. TOM5 cDNA will cross-hybridise with two clones: the genomic clone known as Clone F (Ray et al, 1992, PMB, 19:401-404) and the cDNA clone known as Psy2 (Bartley and Scolnik, 1993, J Biol Chem, 268:25718-25721). Psy2 has 80%

overall nucleotide homology to TOM5 cDNA. The TOM5 cDNA sequence has also been used as a hybridisation probe to identify phytoene synthase clones from other species, including Capsicum annuum which has 83% overall homology (Romer et al, 1993, Biochem Biophys Res Commun, 196:1414-1421) and Arabidopsis thaliana which has 65% overall homology (Scolnik and Bartley, 1994, Plant Physiology, 104:1471-1472).

International application WO91/09128 (EP-A-505405) describes "antisense" and "sense" DNA constructs encoding phytoene synthase and their use to modify the synthesis of carotenoid compounds in plants. These DNA constructs comprise a DNA sequence homologous to some or all of the TOM5 gene, preceded by a transcriptional initiation region operative in plants so that the construct can generate mRNA in plant cells. The DNA construct may encode mRNA which can be translated to give the enzyme produced by the TOM5 gene. International application WO91/09128 describes a method for modifying (inhibiting or promoting) the production of carotenoids in plants by transforming such plants with the aforementioned DNA constructs, and also describes transformed plants (and their descendants) having a modified carotenoid content. Generally, production of the TOM5 enzyme (phytoene synthase) within the plant is enhanced by constructs which contain DNA homologous to the substantially complete gene. For example, promotion of lycopene production may be brought about by inserting one or more functional copies of the TOM5 cDNA, or of the full-length TOM5 gene, under control of a functional plant promoter into a tomato plant to give fruit of a deeper red colour. Fray and Grierson (1993, Plant Mol Biol, 22:589-602) discuss the over-expression of plant phytoene synthase genes in tomato and the effect on colour.

In summary, international application WO91/09128 describes the control of TOM5 gene expression in plants (using recombinant DNA technology) to modify carotenoid metabolism (by increasing or decreasing total carotenoid content of plant parts or increasing/decreasing levels of individual carotenoids). In particular, it describes the modification of colour production and certain other related functions (β-carotene and Vitamin A production; protection against high light intensity damage; control of sporopollenin synthesis and pollen formation/maturation).

In work leading to the present invention we have identified an alternative DNA sequence encoding a phytoene synthase which is useful for

modifying carotenoid metabolism in plants.

According to the present invention, there is provided a DNA sequence encoding melon phytoene synthase. The DNA sequence may be derived from cDNA, from genomic DNA or may be synthesised ab initio.

cDNA clones encoding melon phytoene synthase may be obtained from melon cDNA libraries. Sequences coding for the whole, or substantially the whole, of the mRNA produced by the corresponding gene may thus be obtained. The cDNA so obtained may be sequenced according to known methods.

A cDNA clone encoding phytoene synthase has been isolated from melon. The clone (hereinafter referred to as MEL5) is almost full length and its nucleic acid sequence (with derived amino acid sequence) is shown in Sequence ID-1.

An alternative source of the DNA sequence is a suitable gene encoding melon phytoene synthase. This gene may differ from the corresponding cDNA in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). Oligonucleotide probes or the cDNA clone may be used to isolate the actual phytoene synthase gene(s) by screening melon genomic DNA libraries. Such genomic clones may include control sequences operating in the plant genome. Thus it is also possible to isolate promoter sequences which may be used to drive expression of the enzymes or any other protein. These promoters may be particularly responsive to certain developmental events and environmental conditions. The melon phytoene synthase gene promoters may be used to drive expression of any target gene.

A further way of obtaining a melon phytoene synthase DNA sequence is to synthesise it ab initio from the appropriate bases, for example using the appropriate cDNA sequence as a guide.

Some or all of the melon phytoene synthase sequence may be incorporated into DNA constructs suitable for plant transformation. These DNA constructs may then be used to modify gene expression in plants. "Antisense" or "partial sense" or other techniques may be used to reduce the expression of the phytoene synthase in plant tissue. The levels of phytoene synthase may also be increased; for example, by incorporation of additional enzyme genes. The additional genes may be designed to give either the same or different spatial and temporal patterns of expression in the plant.

According to a further aspect of the invention there is provided a DNA construct comprising some or all of a DNA sequence encoding melon phytoene synthase under the control of a transcriptional initiation region operative in plants, so that the construct can generate RNA in plant cells.

The carotenoid content and related characteristics of plant parts (particularly fruit) may be modified by transformation with a DNA construct according to the invention. The invention also provides plant cells containing such constructs; plants derived therefrom showing modified carotenoid content; and seeds of such plants.

A DNA construct according to the invention may be an "antisense" construct generating "antisense" RNA or a "sense" construct (encoding at least part of the functional enzyme) generating "sense" RNA. "Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). "Sense RNA" is an RNA sequence which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299) or a sense construct encoding and expressing the functional enzyme may be transformed into the plant to over-express the enzyme.

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences

between 100 and 1000 bases in length for downregulation purposes but somewhere in excess of about 1500 bases is preferred for overexpression. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used. The isolation of suitable melon phytoene synthase-encoding sequences is described above. Sequences coding for the whole, or substantially the whole, of the enzyme may thus be obtained. Suitable lengths of this DNA sequences may be cut out for use by means of restriction enzymes. When using genomic DNA as the source of a partial base sequence for transcription it is possible to use either intron or exon regions or a combination of both.

To obtain constructs suitable for expression of melon phytoene synthase in plant cells, the cDNA sequence as found in the enzyme cDNA or the gene sequence as found in the chromosome of the plant may be used. Recombinant DNA constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by annealing and ligating synthetic oligonucleotides or by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator sequences. If antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

In a construct expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and vice versa. The construct will thus encode RNA in a base sequence which is complementary to part or all of the sequence of the enzyme mRNA. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

In a construct expressing sense RNA, the template and coding strands retain the assignments and orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence which is homologous to part or all of the sequence of the mRNA. In constructs which express the functional enzyme, the whole of the coding region of the gene

is linked to transcriptional control sequences capable of expression in plants.

For example, constructs according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (such as the MEL5 cDNA clone) is treated with restriction enzymes to cut the sequence out. The DNA strand so obtained is cloned (if desired, in reverse orientation) into a second vector containing the desired promoter sequence and the desired terminator sequence. Suitable promoters include the 35S cauliflower mosaic virus promoter and the tomato polygalacturonase gene promoter sequence (Bird et al, 1988, Plant Molecular Biology, 11:651-662) or other developmetally regulated fruit promoters. Suitable terminator sequences include that of the Agrobacterium tumefaciens nopaline synthase gene (the nos 3' end).

In a DNA construct according to the invention, the transcriptional initiation region may be derived from any plant-operative promoter. The transcriptional initiation region may be positioned for transcription of a DNA sequence encoding RNA which is complementary to a substantial run of bases in a mRNA encoding phytoene synthase (making the DNA construct a full or partial antisense construct).

The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter (such as fruit-specific promoters), as circumstances require. For example, it may be desirable to modify enzyme activity only during fruit development and/or ripening. Use of a constitutive promoter will tend to affect enzyme levels and functions in all parts of the plant, while use of a tissue specific promoter allows more selective control of gene expression and affected functions (eg fruit colouration). Thus in applying the invention it may be found convenient to use a promoter that will give expression during fruit development and/or ripening. Thus the antisense or sense RNA is only produced in the organ in which its action is required. Fruit development and/or ripening-specific promoters that could be used include the ripening-enhanced polygacturonase promoter (International Patent Publication Number WO92/08798), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7:3315-3320) and the fruit specific 2A11 promoter (Pear et al, 1989, Plant Molecular Biology, 13:639-651).

The DNA constructs of the invention may be inserted into plants to regulate the production of phytoene synthase resulting in modification of carotenoid metabolism. Depending on the nature of the construct, the production of the enzyme may be increased, or reduced, either throughout or at particular stages in the life of the plant. Generally, as would be expected, production of the enzyme is enhanced only by constructs which express RNA homologous to the substantially complete endogenous enzyme mRNAs. Full-length sense constructs may also inhibit enzyme expression. Constructs containing an incomplete DNA sequence shorter than that corresponding to the complete gene generally inhibit the expression of the gene and production of the enzymes, whether they are arranged to express sense or antisense RNA.

A DNA construct of the invention is transformed into a target plant cell. The target plant cell may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. The target plant cell may be selected from any monocotyledonous or dicotyledonous plant species.

Constructs according to the invention may be used to transform any plant using any suitable transformation technique to make plants according to the invention. Both monocotyledonous and dicotyledonous plant cells may be transformed in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Any suitable method of plant transformation may be used. For example, dicotyledonous plants such as tomato and melon may be transformed by Agrobacterium Ti plasmid technology, such as described by Bevan (1984, Nucleic Acid Research, 12:8711-8721) or Fillatti et al (Biotechnology, July 1987, 5:726-730). Such transformed plants may be reproduced sexually, or by cell or tissue culture.

Examples of genetically modified plants according to the present invention include all fruit-bearing plants (such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, peppers, chillies, paprika). For example, the fruit of such plants may be made more attractive (or at least interesting) by inducing or intensifying a red colour therein. Other plants that may be modified by the process of the

invention include tubers such as radishes, turnips and potatoes, as well as cereals such as maize (corn), wheat, barley and rice. Flowers and ornamental grasses may also be modified.

Use of the novel melon phytoene synthase constructs allows modification of enzyme activity and thus provides a method for modification of carotenoid biosynthesis. The overall level of phytoene synthase activity and the relative activities of the individual enzymes of the carotenoid pathway affect the development and final form of carotenoid content in the plant and thus determine certain characteristics of the plant parts. Modification of phytoene synthase activity can therefore be used to modify various aspects of plant (including fruit) quality. The activity levels of the phytoene synthase enzyme may be either increased or reduced during development depending on the characteristics desired for the modified plant. Enhancing expression of phytoene synthase will tend to increase production of certain carotenoids, and inhibiting expression will tend to decrease production. Phytoene synthase may also be expressed in cells, tissues and organisms that do not normally produce the enzyme.

For example, the down-regulation or over-expression of phytoene synthase may be used to modify (alter or enhance) fruit colour by altering pigment levels. This is particularly useful for modifying fruit and vegetable colour but may equally be applied to leaves and other organs.

Carotenoid content (and hence plant characteristics) may be modified to a greater or lesser extent by controlling the degree of the sense or antisense mRNA production in the plant cells. This may be done by suitable choice of promoter sequences, or by selecting the number of copies or the site of integration of the DNA sequences that are introduced into the plant genome. For example, the DNA construct may include more than one DNA sequence encoding phytoene synthase or more than one recombinant construct may be transformed into each plant cell.

It is also possible to modify the activity of phytoene synthase while also modifying the activity of one or more other enzymes. For example, the other enzymes may be involved in cell metabolism or in fruit development and ripening. Cell wall metabolising enzymes that may be modified in combination with phytoene synthase include but are not limited to: pectin esterase, polygalacturonase, β-galactanase, β-glucanase. Other enzymes involved in fruit development and ripening that may be modified in

combination with phytoene synthase include but are not limited to: ethylene biosynthetic enzymes, other carotenoid biosynthetic enzymes, carbohydrate metabolism enzymes including invertase.

Several methods are available for modification of the activity of phytoene synthase in combination with other enzymes. For example, a first plant may be individually transformed with a phytoene synthase construct and then crossed with a second plant which has been individually transformed with a construct encoding another enzyme. As a further example, plants may be either consecutively or co-transformed with phytoene synthase constructs and with appropriate constructs for modification of the activity of the other enzyme(s). An alternative example is plant transformation with a phytoene synthase construct which itself contains an additional gene for modification of the activity of the other enzyme(s). The phytoene synthase constructs may contain sequences of DNA for regulation of the expression of the other enzyme(s) located adjacent to the phytoene synthase sequences. These additional sequences may be in either sense or antisense orientation as described in International patent application publication number WO93/23551 (single construct having distinct DNA regions homologous to different target genes). By using such methods, the benefits of modifying the activity of phytoene synthase may be combined with the benefits of modifying the activity of other enzymes.

According to a third aspect of the invention, there is provided a method for modifying the production of carotenoids in plants by transforming such plants with DNA adapted to modify carotenoid biosynthesis and growing such transformed plants or their descendants followed by selection of plants having plant parts (for example leaves, petals or fruit) of modified carotenoid content. Suitable DNA comprises constructs according to the invention which may be adapted to enhance the production of one or more carotenoids or to inhibit such production by the plant.

In this way, plants can be generated which have modified colour due to promotion or inhibition of the pathways of carotenoid biosynthesis. In particular, phytoene synthase constructs may be used to promote or inhibit the production of the red colour associated with lycopene. Similar colour modifications may be possible using traditional plant breeding techniques, but the present invention provides a means of transferring the trait into elite lines without a prolonged breeding programme which might alter other

traits at the same time. Promotion of lycopene production (eg by sense over-expression constructs) may produce fruit of a deeper red colour, which may appear more appetising to the consumer. The invention may also be used to introduce a red colour into parts of plants other than the fruit. For example, promotion of lycopene may be brought about by inserting one or more functional copies of the gene cDNA, or of the full-length gene, under control of a promoter functional in plants. If phytoene synthase is naturally expressed in the plant, the promoter may be selected to give a higher degree of expression than is given by the natural promoter.

As well as colour production, other important functions may be modified by the process of the invention. Thus β-carotene (a precursor of Vitamin A) and other carotenoids are important to human health, and have been claimed to have a protective effect against certain diseases. Food plants may be modified by transformation with the constructs of the invention so that they have a higher content of such compounds: or other plants may be so modified, so that they can act as a source from which such compounds can be extracted. Carotenoids are also believed to have a role in protecting plants against high light intensity damage, so plants with a higher content of such compounds may be of value in combating the effects of any global climate change.

As already discussed, plants produced by the process of the invention may also contain other recombinant constructs, for example constructs having other effects on fruit ripening. For example fruit of enhanced colour according to the invention may also contain constructs inhibiting the production of enzymes such as polygalacturonase and pectinesterase, or interfering with ethylene production. Fruit containing both types of recombinant construct may be made either by successive transformations, or by crossing two varieties that each contain one of the constructs, and selecting among the progeny for those that contain both.

The invention will now be described by way of example only, with reference to the drawings in which:

Figure 1 is a diagram of the carotenoid biosynthesis pathway;

Figure 2 illustrates a sense construct; and,

Figure 3 illustrates an antisense construct.

The following gene sequences are provided herewith:

Sequence ID-1 is the nucleotide sequence of MEL5;

Sequence ID-2 is the sequence of TOM 5, the equivalent sequence from tomato.

EXAMPLE 1

cDNA clone encoding melon phytoene synthase

The MEL5 cDNA clone was isolated from a ripening related cDNA library derived from climacteric melon fruit, using the tomato phytoene synthase cDNA (TOM5, see Ray et al, 1992 or EP-A-505405) as a heterologous probe. Genomic Southern analysis indicated that the MEL5 clone is encoded by a single copy or small number of genes (genomic melon DNA was digested with BamHI, EcoRV, BamHI/EcoRV and hybridised to radiolabelled MEL5 insert, followed by washing up to 0.1xSSC, 0.1% SDS for 30 minutes at 42°C or 50°C).

Northern analysis of RNA extracted from a ripening series of fruit and from roots, leaves and flower petals was used to examine the pattern of expression of this mRNA. Total RNA was extracted from different ripening stages of fruit (green fruit not producing ethylene; green/orange fruit producing 5-7 nl/g/h ethylene; orange fruit producing 3-6 nl/g/h and very orange fruit producing approximately 1 nl/g/h ethylene). Total RNA and poly(A)$^+$ RNA from fruit, leaves, petals and roots was hybridised to radiolabelled MEL5 insert. MEL5 hybridised to a transcript of 1.7 kB which was present at all stages of fruit ripening, in petals and in trace amounts in leaves and roots. The cDNA was 1.36 kB in size, and sequence analysis of the longest open reading frame demonstrated 68% identity at the nucleotide level and 83.1% similarity at the amino acid level with the tomato fruit phytoene synthase. The MEL5 cDNA clone was not full length but the 5' end was isolated using a PCR approach, and subsequently fully sequenced (Sequence ID-1).

EXAMPLE 2

Construction of antisense RNA vectors with the CaMV 35S promoter

A vector is constructed using the sequences corresponding to a fragment of the insert of a melon phytoene synthase cDNA. This fragment is synthesised by polymerase chain reaction using synthetic primers. The ends of the fragment are made flush with T4 polymerase and it is cloned into the vector pJR1 which has previously been cut with SmaI. pJR1 (Smith et al, 1988, Nature, 334:724-726) is a Bin19 (Bevan, 1984, Nucleic Acids Research, 12:8711-8721) based vector, which permits the expression of the antisense

RNA under the control of the CaMV 35S promoter. This vector includes a nopaline synthase (nos) 3' end termination sequence.

Alternatively a vector is constructed using a restriction fragment obtained from a melon phytoene synthase cDNA and cloned into the vectors GA643 (An et al, 1988, Plant Molecular Biology Manual A3: 1-19) or pDH51 (Pietrzak et al, 1986, Nucleic Acids Research, 14:5875-5869) which has previously been cut with a compatible restriction enzyme(s). A restriction fragment from the phytoene synthase/pDH51 clone containing the promoter, the phytoene synthase fragment and other pDH51 sequence is cloned into SLJ44026B or SLJ44024B (Jones et al, 1990, Transgenic Research, 1) or Bin19 (Bevan, 1984, Nucleic Acids Research, 12:8711-8721) which permits the expression of the antisense RNA under control of the CaMV 35S promoter. This procedure is illustrated in Figures 2 and 3.

After synthesis of the vector, the structure and orientation of the sequences are confirmed by DNA sequence analysis.

EXAMPLE 3

Construction of antisense RNA vectors with a fruit enhanced promoter.

Referring to Figures 2 and 3 the fragment of the melon phytoene synthase cDNA that was described in Example 2 is also cloned into the vector pJR3. pJR3 is a Bin19 based vector, which permits the expression of the antisense RNA under the control of the tomato polygalacturonase (PG) promoter. This vector includes approximately 5 kb of promoter sequence and 1.8 kb of 3' sequence from the PG promoter separated by a multiple cloning site.

After synthesis, vectors with the correct orientation of the phytoene synthase sequences are identified by DNA sequence analysis.

Alternative fruit enhanced promoters (such as E8 or 2A11) are substituted for the polygalacturonase promoter in pJR3 to give alternative patterns of expression.

EXAMPLE 4

Construction of truncated sense RNA vectors with the CaMV 35S promoter

The fragment of the phytoene synthase cDNA that was described in Example 2 is also cloned into the vectors described in Example 2 in the sense orientation.

After synthesis, the vectors with the sense orientation of the phytoene synthase sequence are identified by DNA sequence analysis.

EXAMPLE 5

Construction of truncated sense RNA vectors with fruit-enhanced promoter.

The fragment of the phytoene synthase cDNA that was described in Example 3 is also cloned into the vectors described in Example 3 in the sense orientation.

After synthesis, the vectors with the sense orientation of the phytoene synthase sequence are identified by DNA sequence analysis.

EXAMPLE 6

Construction of an over-expression vector using the CaMV35S promoter

The complete sequence of a melon phytoene synthase cDNA containing a full open-reading frame is inserted into the vectors described in Example 2.

EXAMPLE 7

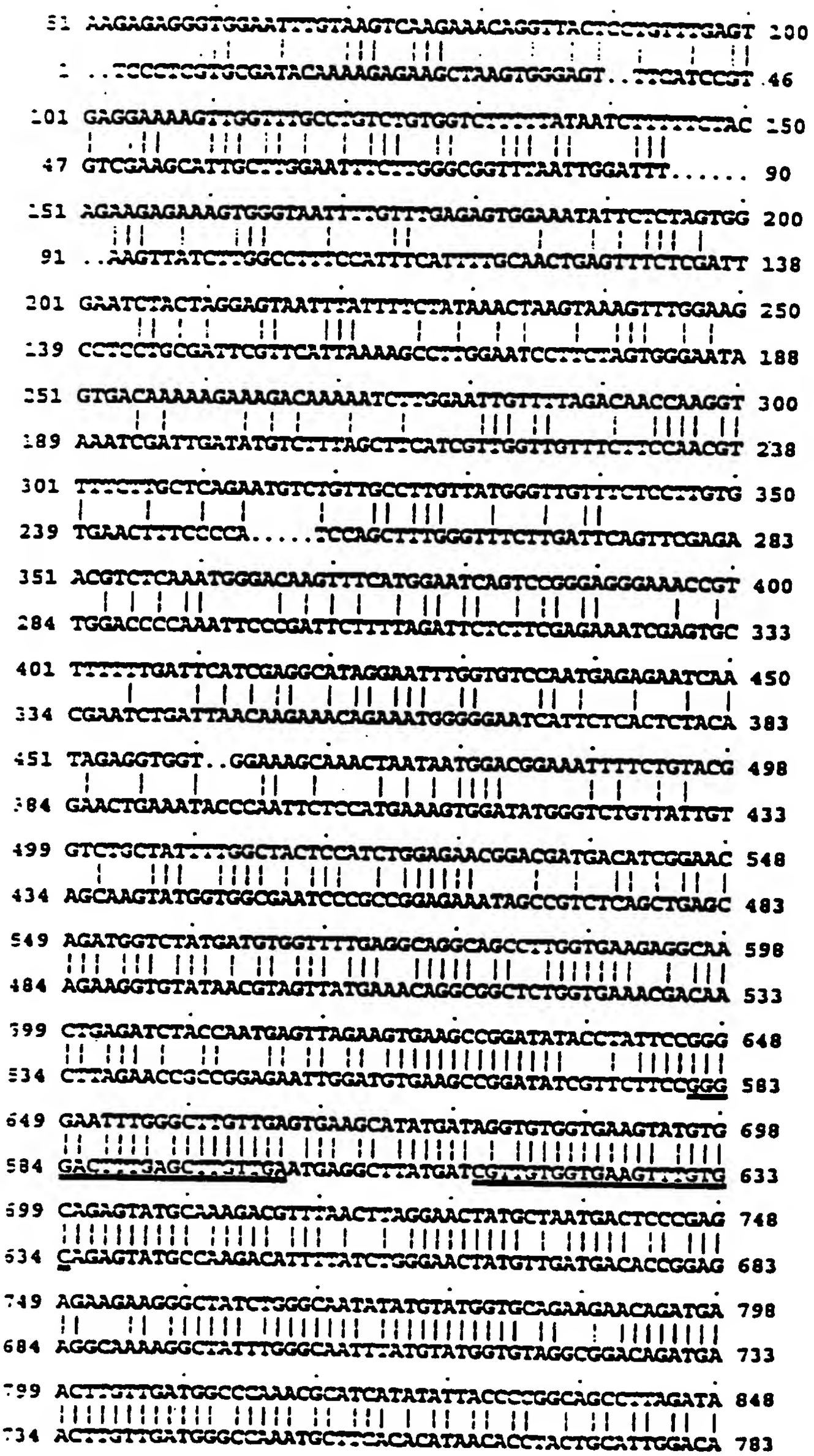
Construction of an over-expression vector using a fruit-enhanced promoter

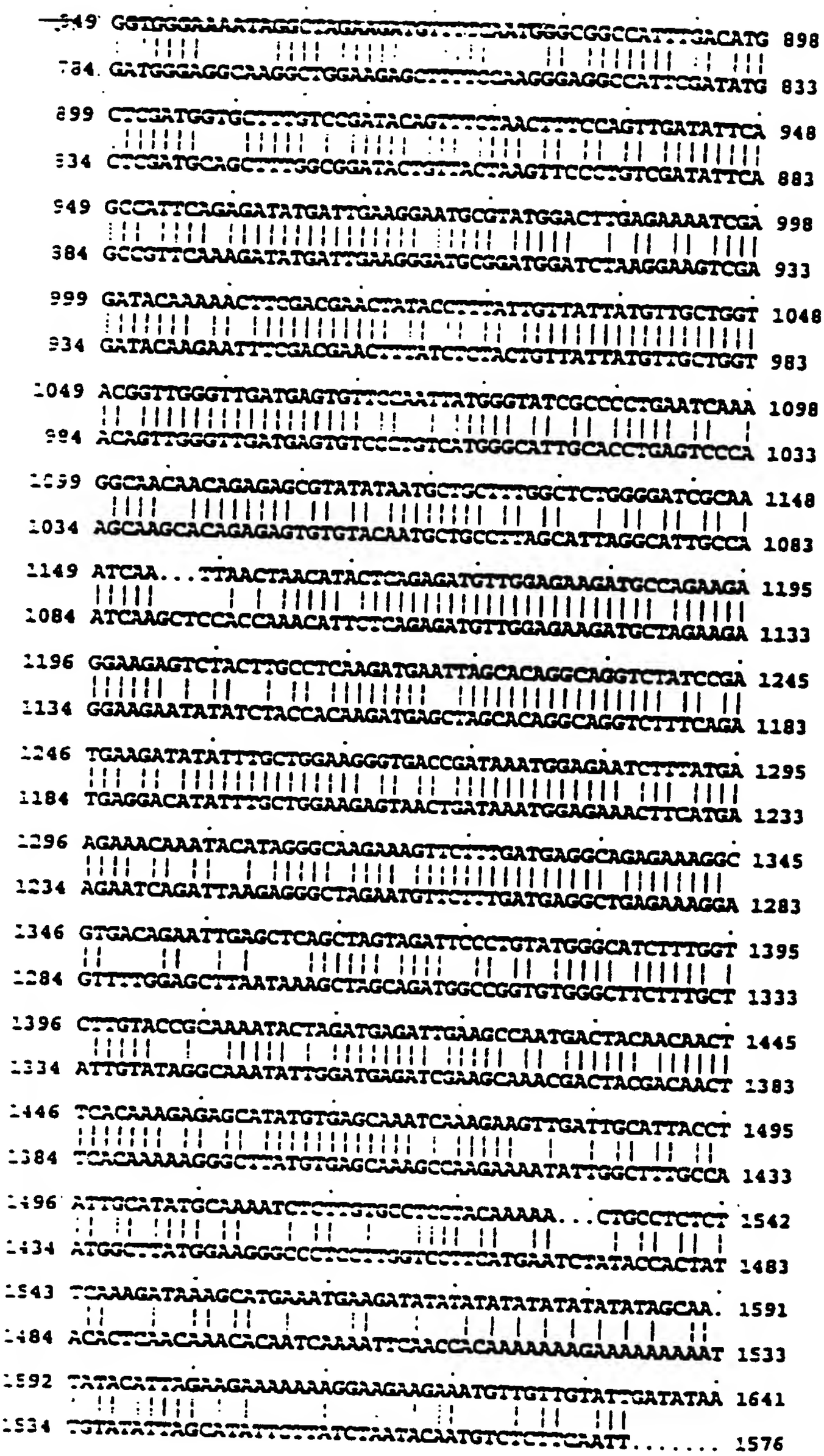
The complete sequence of a melon phytoene synthase cDNA containing a full open-reading frame is inserted into the vectors described in Example 3 (pJR3 or alternatives with different promoters).

EXAMPLE 8

Generation of transformed plants

Vectors are transferred to Agrobacterium tumefaciens LBA4404 (a micro-organism widely available to plant biotechnologists) and are used to transform tomato plants. Transformation of tomato stem segments follow standard protocols (e.g. Bird et al, 1988, Plant Molecular Biology, 11:651-662). Transformed plants are identified by their ability to grow on media containing the antibiotic kanamycin. Plants are regenerated and grown to maturity. Ripening fruit are analyzed for modifications to their carotenoid content.





CLAIMS

1. A DNA encoding melon phytoene synthase.

2. The cDNA encoding melon phytoene synthase having the nucleotide sequence ID-1 .

3. A DNA construct comprising some or all of a DNA sequence encoding melon phytoene synthase under the control of a transcriptional initiation region operative in plants, so that the construct can generate RNA in plant cells.

4. A DNA construct as claimed in claim 3, in which the said DNA sequence is in antisense orientation.

5. A DNA construct as claimed in claim 4, in which the said DNA is a full length equivalent of the melon phytoene synthase gene.

6. A DNA construct as claimed in claim 4, in which the said DNA is a partial sequence containing a sufficient number oif nucleotides to effect inhibition of the expression of the endogenous phytoene synthase gene of a plant.

7. A DNA construct as claimed in claim 3, in which the said DNA is in sense orientation.

8. A DNA construct as claimed in claim 7, in which the said DNA is a full length equivalent of the melon phytoene synthase gene.

9. A DNA construct as claimed in claim 7, in which the said DNA is a partial sequence of the melon phytoene synthase gene.

10. A DNA construct as claimed in any of claims 3 to 9, in which said DNA comprises a sequence of at least 10 nucleotides for transcription into RNA.

11. A DNA construct as claimed in claim 10, in which the said DNA comprises at least 35 nucleotides.

12. A method of modifying the production of carotenoids in a plant comprising transforming said plant with DNA adapted to modify carotenoid biosynthesis and growing said transformed plants or their descendants followed by selection of plants having plant parts of modified carotenoid content.

13. A method as claimed in claim 12, in which the plant is modified to enhance the production of one or more carotenoids or to inhibit such production by the plant.

14. A plant have a modified ability to produce carotenoids.

Figure 1

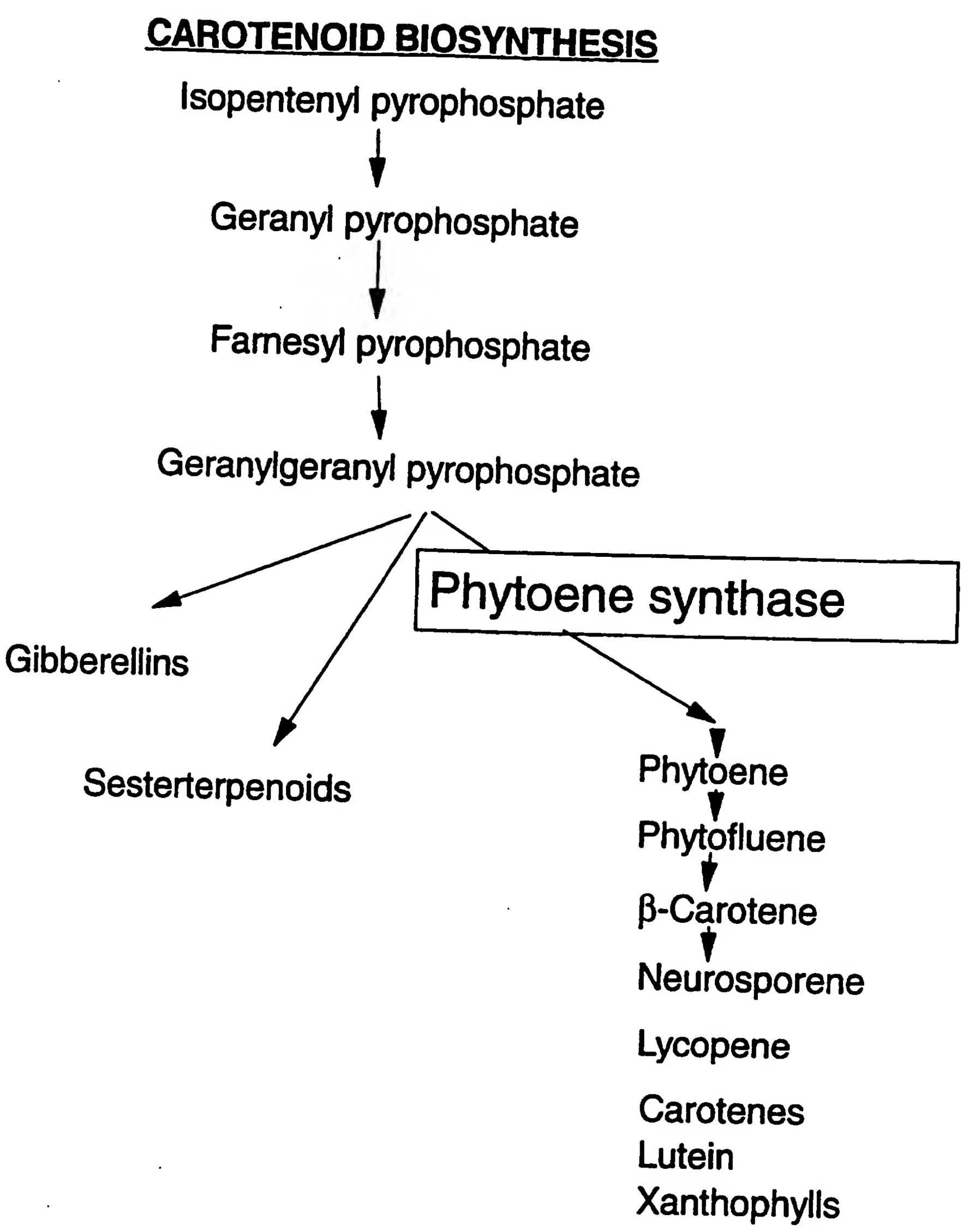

CAROTENOID BIOSYNTHESIS
Isopentenyl pyrophosphate
Geranyl pyrophosphate
Farnesyl pyrophosphate
Geranylgeranyl pyrophosphate
Phytoene synthase
Gibberellins
Sesterterpenoids
Phytoene
Phytofluene
β-Carotene
Neurosporene
Lycopene
Carotenes
Lutein
Xanthophylls

Figure 2

MEL5 Sense Construct

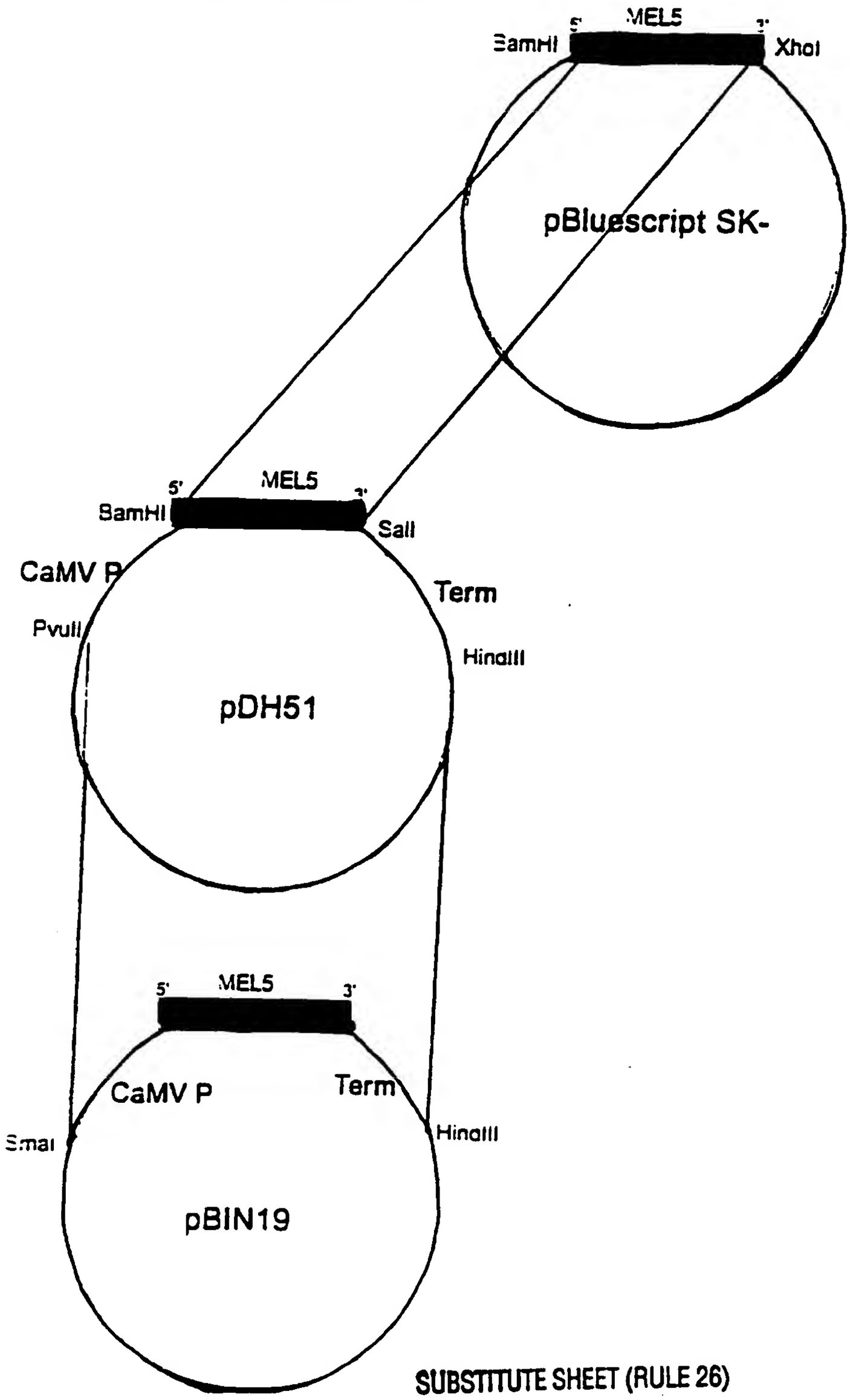
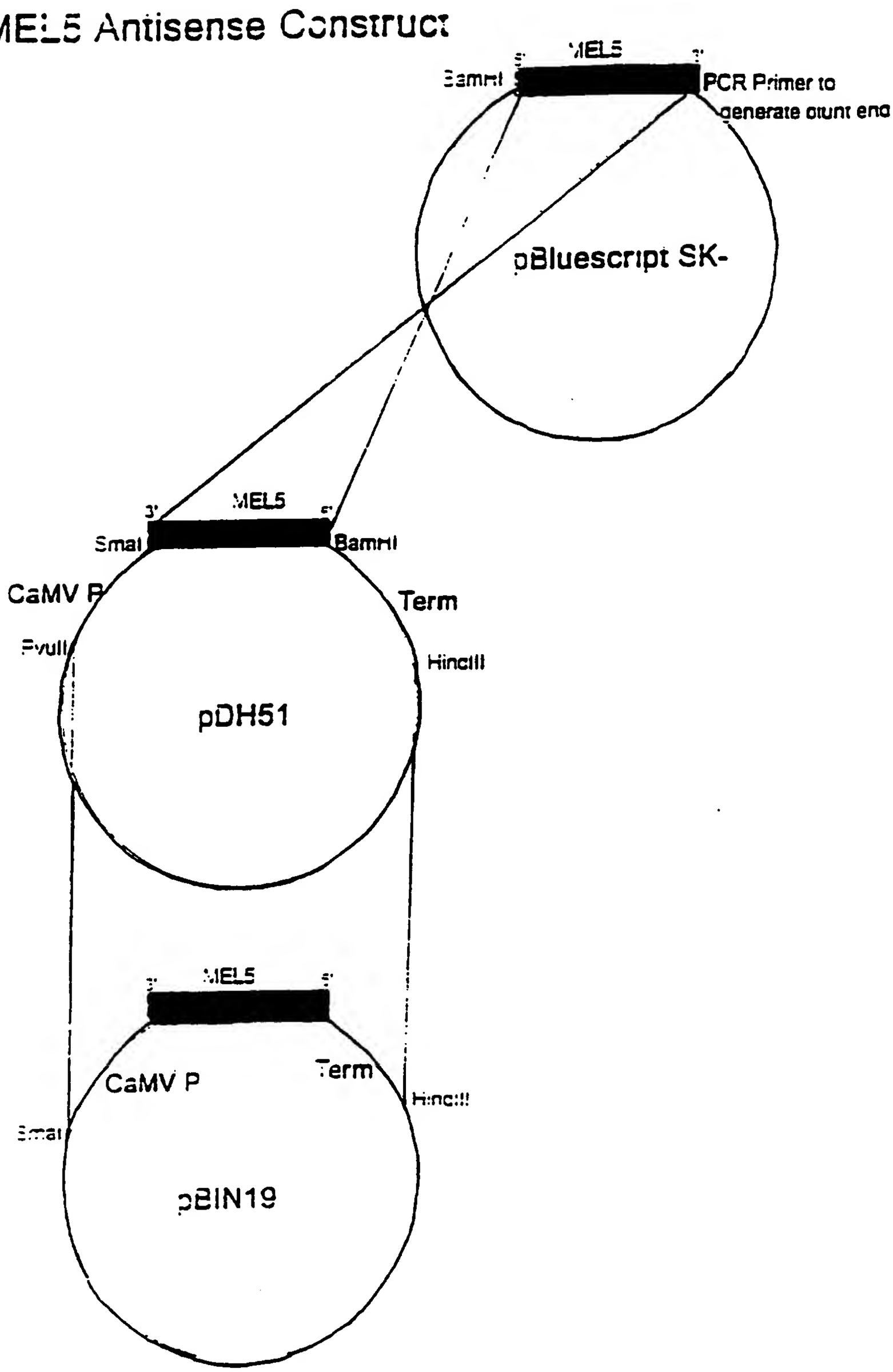


Figure 3


MEL5 Antisense Construct
MEL5
BamHI
PCR Primer to
generate blunt end
pBluescript SK-
3'
MEL5
5'
SmaI
BamHI
CaMV P
Term
EvuII
HincIII
pDH51
MEL5
Term
CaMV P
HincIII
SmaI
pBIN19

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/52, 15/82, A01H 5/00

A3

(11) International Publication Number: WO 96/02650

(43) International Publication Date: 1 February 1996 (01.02.96)

(21) International Application Number: PCT/GB95/01603

(22) International Filing Date: 6 July 1995 (06.07.95)

(30) Priority Data:
9414505.9 18 July 1994 (18.07.94) GB
9419081.6 22 September 1994 (22.09.94) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KARVOUNI, Zoi [GR/GR]; Vioryl, 36 Viltaniotis Street, GR-145 64 Kifissia (GR). JOHN, Isaac [PK/GB]; University of Nottingham, Dept. of Physiology and Environmental Science, Sutton Bonington, Loughborough LE12 5RD (GB). TAYLOR, Jane [GB/GB]; Lancaster University, Institute of Environmental and Biological Sciences, Division of Biological Sciences, Lancaster LA1 4YQ (GB). WATSON, Colin [GB/US]; University of Arizona, Dept. of Plant Sciences, Tucson, AZ 85721 (US). TURNER, Andrew [GB/GB]; Scotgen Limited, 2 Lillydron Avenue, Aberdeen AB9 2TN (GB). GRIERSON, Donald [GB/GB]; University of Nottingham, School of Agriculture, Dept. of Plant Physiology, Sutton Bonington, Loughborough LE12 5RD (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG12 6YA (GB).

(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(88) Date of publication of the international search report:
13 February 1997 (13.02.97)

(54) Title: DNA, CONSTRUCTS, CELLS AND PLANTS DERIVED THEREFROM

(57) Abstract

A DNA encoding melon phytoene synthase.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 95/01603

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT JOURNAL, vol. 2, no. 3, 1993 pages 343-349, BRAMLEY, P., ET AL. 'BIOCHEMICAL CHARACTERIZAION OF TRANSGENIC TOMATO PLANTS IN WHICH CAROTENOID SYNTHESIS HAS BEEN INHIBITED THROUGH EXPRESSION OF ANTISENSE RNA TO PTOM5' see the whole document --- -/--	10-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 1996	20. 03. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/GB 95/01603

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT JOURNAL, vol. 4, no. 5, 1993 pages 833-840, MISAWA, N., ET AL. 'FUNCTIONAL EXPRESSION OF THE ERWINIA UREDOVORA CAROTENOID BIOSYNTHESIS GENE CRTL IN TRANSGENIC PLANTS SHOWING AN INCREASE OF BETA-CAROTENE BIOSYNTHESIS ACTIVITY AND RESISTANCE TO THE BLEACHING HERBICIDE NORFLURAZON' see the whole document	12-14
X	WO,A,91 09128 (ICI PLC) 27 June 1991 see the whole document	10-14
X	WO,A,91 13078 (AMOCO CORP) 5 September 1991 see the whole document	12-14
X	PLANT MOLECULAR BIOLOGY, vol. 22, 1993 pages 589-602, FRAY, R.G., ET AL. 'IDENTIFICATION AND GENETIC ANALYSIS OF NORMAL AND MUTANT PHYTOENE SYNTHASE GENES OF TOMATO BY SEQUENCING, COMPLEMENTATION AND CO SUPPRESSION' see the whole document	10-14
P,X	PLANT MOLECULAR BIOLOGY, vol. 27, no. 6, March 1995 pages 1153-1162, KARVOUNI, Z., ET AL. 'ISOLATION AND CHARACTERISATION OF A MELON CDNA CLONE ENCODING PHYTOENE SYNTHASE' see the whole document	1,2
A	WO,A,92 16635 (ICI PLC) 1 October 1992 see the whole document	1-14

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/GB 95/01603

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9109128	27-06-91	AU-B-	645534	20-01-94
		AU-B-	6893891	18-07-91
		EP-A-	0505405	30-09-92
		EP-A-	0699765	06-03-96
		JP-T-	5502160	22-04-93
		US-A-	5304478	19-04-94
WO-A-9113078	05-09-91	EP-A-	0471056	19-02-92
		JP-T-	5504686	22-07-93
WO-A-9216635	01-10-92	AU-B-	1369092	21-10-92
		BR-A-	9205770	07-06-94
		CA-A-	2106091	15-09-92
		EP-A-	0619844	19-10-94
		JP-T-	6505871	07-07-94